The Relationship between Shrinkage of Hide and the Crystal-Liquid Transition of Collagen

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Abstract

A shrinkage measurement is employed to determine the effect of temperature on hide specimens containing small quantities of a compatible diluent such as water, ethylene glycol, formamide, and phenol. The temperature at which contraction takes place is shown to depend upon the concentration of diluent and is found to increase as the diluent concentration is decreased. This behavior of hide is identical to that reported by Garrett and Flory [*Nature,* **177,** 176 (1956)] for a tendon collagen-ethylene glycol system from data obtained using a dilatometer. The observed shrinkage of hide is attributed to a melting phenomenon and is an outward manifestation of the collapse of the helical structure of collagen. The theory of melting-point depression as applied to semicrystalline polymers is employed in treating the shrinkage temperature-diluent data. The effect of various tanning agents on the crystal-liquid transition temperature of the hide-water systems is discussed.

Hide specimens when heated while immersed in water or other polar substances undergo a marked dimensional change, namely, shrinkage, at a specific temperature which is commonly referred to as the shrinkage temperature. This behavior has been variously described as due to degradation, denaturation, rearrangement, gelation, or various combinations of each of these processes. Garrett and Flory observed that tendon collagen containing a limited quantity of ethylene glycol abruptly increased in volume at a specific temperature which depended on the glycol content. This behavior is characteristic of semicrystalline polymeric substances which undergo dimensional changes at the melting point of the crystallites. Tem-

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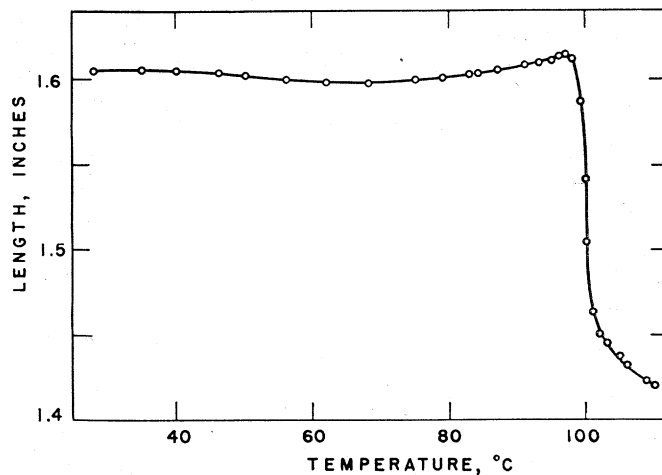
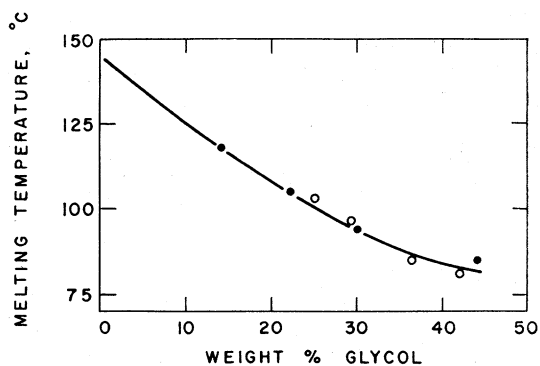Fig. 1. Plot of length vs. temperature for plain cowhide containing 29.5% by weight of ethylene glycol.

Fig. 2. Plot of apparent melting (shrinkage) temperature of hide vs. weight per cent glycol. Dilatometric melting-point data of Garrett and Flory (●); data obtained by shrinkage measurements (O).

perature of melting is lowered by addition of a compatible diluent to the polymer and the amount of depression is proportional to the amount of diluent present. Garrett and Flory were able to show that the theory of melting point depression as applied to semicrystalline polymers was also applicable to the collagen-ethylene glycol system;
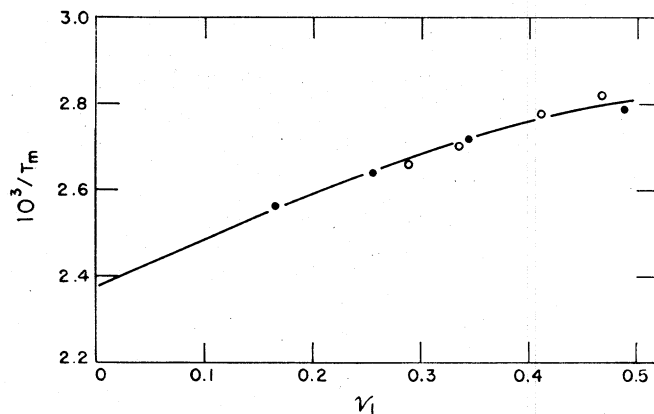
Fig. 3. Plot of reciprocal melting temperature vs. volume fraction of diluent, v_1, for the ethylene glycol–cowhide system (O), and for the ethylene glycol–collagen system (●).

thus the principal event that occurs on heating the tendon collagen system is a crystal-liquid transition.

The purpose of the present investigation was: (1) to determine the adequacy of substituting simple shrinkage temperature measurements for the more tedious dilatometric measurements previously employed in studying the transitional behavior of collagen; (2) to replace tendon collagen with hide specimens in such studies; (3) to extend the measurements to other compatible diluents including water; and (4) to determine the applicability of the method to tanned hide specimens.

In order to verify the first two points, the shrinkage temperatures of limed-hide specimens containing varying amounts of ethylene glycol were determined. All shrinkage measurements were made on hide specimens that had been vacuum dried prior to conditioning in an atmosphere of the diluent. The specimens were immersed in mercury to prevent loss of diluent during testing. Figure 1 shows a typical shrinkage temperature determination curve. Shrinkage occurred at a specific temperature which was reproducible to ±1°C. in separate runs.

The shrinkage temperatures of hide specimens containing 25.0 to 42.0% by weight of ethylene glycol are given in Figure 2. The shrinkage temperature decreases with increasing ethylene glycol

content in accordance with the concept of a melting process. Also included in the figure are the data of Garrett and Flory obtained for tendon collagen. The agreement between the intact hide data and the tendon collagen data is very good considering the fact that both the origin and treatment of the samples studied were quite different.

Flory has shown that the melting point of semicrystalline polymer systems is depressed by diluents as demanded by the relationship obtained by the application of thermodynamic equilibrium to phase transitions. The expression used in treating the experimental data was the following:

$$1/T_m - 1/T_{m0} = (R/\Delta H_u)(V_u/V_1)(v_1 - x_1 v_1^2)$$

T_m—melting point of polymer-diluent system; T_{m0}—melting point of pure polymer; v_1—volume fraction of diluent; V_u and V_1—molar volume of polymer and diluent, respectively; x_1—interaction parameter; ΔH_u—heat of fusion of the crystalline material; and R—gas law constant.

If the reciprocal of the shrinkage temperature is plotted against volume fraction of diluent for the hide system under investigation and the curve extrapolated to zero concentration, the melting point of crystallites should be obtained. A plot of the reciprocal of the absolute shrinkage temperature against the volume fraction of ethylene glycol is shown in Figure 3. Also shown in the graph are similar data obtained by Garrett and Flory with a dilatometer using
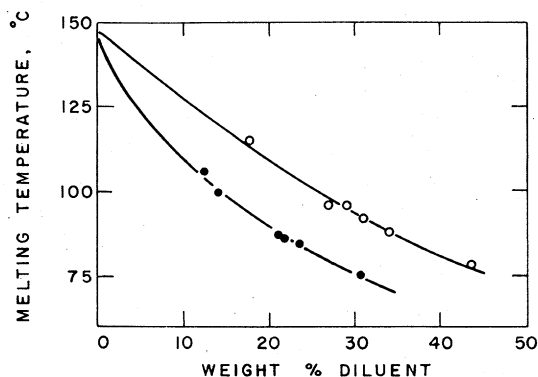

Fig. 4. Plot of apparent melting (shrinkage) temperature of hide vs. weight per cent formamide (●); phenol (○).
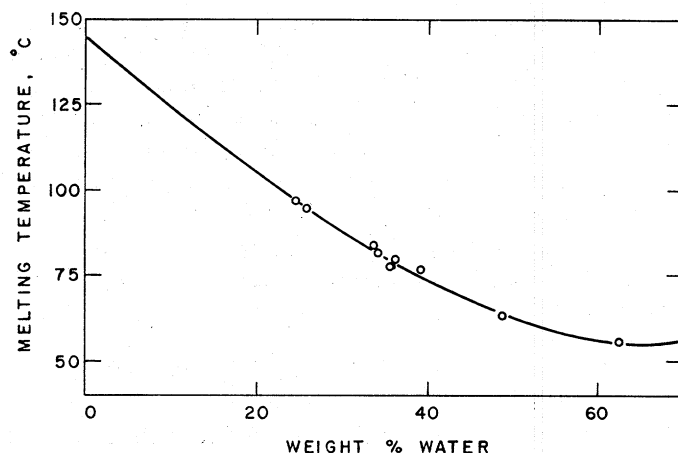
Fig. 5. Plot of apparent melting (shrinkage) temperature of hide vs. weight per cent water.

collagen and ethylene glycol. Extrapolation of the curve to zero diluent content yields a value of about 145°C. for the melting point of the crystallites in undiluted cowhide, the same as reported for collagen. No real melting of the crystallites of vacuum dried cowhide could be detected experimentally even at temperatures considerably above 145°C. The bonding forces within the crystalline regions are so strong that decomposition takes place instead of melting.

Figure 4 gives results obtained using phenol and formamide as diluents. The phenol-hide data are represented by the upper curve, the formamide-hide data by the lower curve. Like the ethylene glycol system, these show a decrease in the shrinkage or melting temperature with increase in diluent concentration. It should be emphasized that the diluent employed must be able to associate in some manner with the polypeptide chain or chains. They must contain a group or groups that can interact with a group or groups contained on or within the collagen molecule. For example, phenol is able to act as a diluent while toluene is completely inert. Extrapolation of the melting (shrinkage) temperature to zero diluent content for all the above-mentioned diluents yielded a value of about 145°C. for the theoretical melting temperature of vacuum dried bovine collagen.

The variation in the shrinkage temperature for unmodified hide containing 25 to 62% by weight of water is shown in Figure 5. Like

the other diluent systems, the shrinkage temperature decreased as the diluent content increased. For example, the hide specimen containing 24.5% water contracted at 97°C., while the one containing 62% water contracted at 56°C. The latter temperature was the same as that obtained when the specimen was completely immersed in water. Apparently the maximum amount of water that is effective in lowering the melting temperature of bovine collagen is about 62%. Specimens taken from different portions of the same limed hide and from other limed hides (including calf skin) in general gave the same melting temperature (56°C.) when completely solvated with water.

These observations indicate that the shrinkage of hide is a melting phenomenon and an outward manifestation of the collapse of the helical structure of collagen. If this is true, then the temperature at which shrinkage occurs should be very nearly that observed for the breakup of collagen helices suspended in aqueous solution. Doty and co-workers reported a value of 37°C. for the so-called denaturation temperature of soluble collagens in a citrate buffer medium (pH 3.7). The shrinkage temperature of limed hide in the same medium was about 3 to 6° higher indicating the close similarity between shrinkage and melting or molecular rearrangement of the collagen helix.

The heat of fusion of the collagen crystallites in each of the four diluent systems investigated is shown in Table I. These values were calculated using the equation previously given. The corresponding values for the entropy of fusion of the crystallites were calculated from the thermodynamic relationship: entropy of fusion is equal to heat of fusion divided by the melting point of the crystallites. Examination of the ΔH_u data shows similar values for the diluents, ethylene glycol, formamide, and phenol, while the value for the water system was almost four times greater. According to the method, heats of fusion obtained using different solvents should be the same. The reason for this discrepancy is that a portion of the water taken up by the hide enters into the crystallites and, therefore, invalidates the thermodynamic relationship employed. Only a portion of the sorbed water is available to act as diluent.

The corresponding values for the entropy of fusion, ΔS_u, were quite similar for the diluents ethylene glycol, formamide, and phenol. These values should be the same if the number of configurational

TABLE I
Heat of Fusion of the Collagen Crystallites

Diluent	$T_{m}0$ °C.	ΔH_u cal./mole	ΔS_u cal./ deg./mole	cal./ deg./bond
Ethylene glycol	145	2200	5.3	3.6
Formamide	145	1600	3.8	1.9
Phenol	145	1800	4.4	2.2
Water	145	7200	—	—

arrangements of the chain segments on melting were identical in the system investigated. The entropy of fusion for most polymers reported are in the range 1.5 to 2.0 cal./deg./bond. Shown in the last column are the corresponding values for the hide system assuming that two bonds per residue were freed on melting.

These studies on untanned hide show that the shrinkage temperature can be elevated quite simply by limiting the amount of diluent that can solvate, that is, associate with the polypeptide chains of hide. One of the well-known effects that a tanning agent has on hide substance is to elevate the shrinkage temperature as measured immersed in water or other diluent systems. Thus a tanning agent might be considered as a substance that reduces the availability of the polypeptide chains to the shrinkage medium. This will, in general, be true regardless of the types of tanning agents employed. It should be emphasized again that the tanning agent must itself become associated with the polypeptide, either through hydrogen bonds, covalent bonds, or electrostatic bonds. Incorporation of a completely unassociated hydrophobic material in unmodified hide substance would only exude out or sweat out and any blocking action toward a diluent would be of very short duration.

A preliminary investigation of the melting-point depression in hide specimens that had been tanned with a vegetable tannin and formaldehyde was undertaken. Water was the diluent used. Like the unmodified hide, the shrinkage temperature of the tanned specimens decreased with increasing water content. Diluents other than water would be expected to exhibit a similar behavior.

Figure 6 shows the data obtained on vegetable-tanned samples of commercial upholstery leather. The shrinkage temperatures ranged
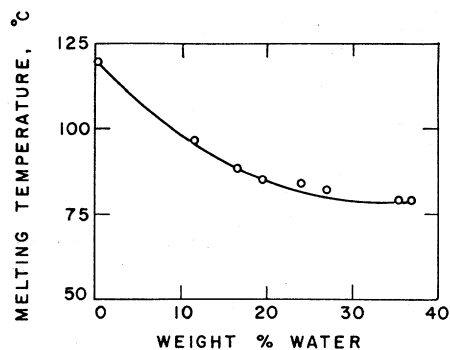
Fig. 6. Plot of apparent melting (shrinkage) temperature of vegetable-tanned hide
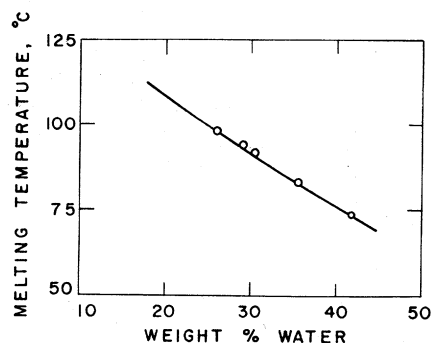vs. weight per cent water.

Fig. 7. Plot of apparent melting (shrinkage) temperature of formaldehyde-tanned
hide vs. weight per cent water.

from 97°C. with 11.5% water to 79°C. with 37% water. The
shrinkage temperatures of vegetable-tanned hide specimens at low
water contents are lower than those found for the unmodified hide-
water system. For example, a tanned specimen containing 25%
water was 13° lower than that of unmodified hide of similar water
content. The reason for this might be that vegetable tannin itself
could act as a diluent, probably because of a loose association of its
polar groups with the polypeptide chains. This diluent action of
vegetable tannin was verified experimentally. It was found that
vacuum dried vegetable tanned hide on heating underwent an un-
mistakable shrinkage at about 120°C. The shrinkage temperature

was not nearly as well defined or reproducible as the other shrinkage measurements. It appeared to have occurred over a temperature range, indicating that in the tanned specimens used the tanning agent was not distributed uniformly throughout the hide substance. When immersed in water, the vegetable-tanned specimen shrank 22° higher than the untanned-hide specimen. The reason for this has already been discussed.

Figure 7 shows similar data for formaldehyde-tanned hide. The melting temperatures ranged from 90°C. for a specimen containing 26% water to 73° for 40% water. When immersed in water its shrinkage temperature was also 73°. The effective water content of the immersed formaldehyde-tanned hide was about 23% less than that obtained with unmodified hide. Dry formaldehyde tanned specimens of hide when heated at temperatures as high as 170°C. showed no appreciable shrinkage. This behavior was quite unlike that of the vegetable-tanned samples. Apparently the mode of attachment of the formaldehyde to the polypeptide chains is different from that of vegetable tannin. This is not unexpected as formaldehyde is known to form covalent crosslinks. Since such crosslinks are relatively short and fixed compared with those of a vegetable tannin, the formaldehyde cannot act as a diluent.

In conclusion, it would appear from the results presented that a simple shrinkage measurement is suitable for studying the transitional behavior of collagen. The behavior of untanned hide when subjected to heat in the presence of small amounts of compatible diluents is analogous to the depression of the melting point of any crystalline substance by a diluent. The macroscopic shrinkage of hide is an outward manifestation of the melting of the collagen helices. The results obtained from tanned hide indicate that tanning agents reduce the amount of water that can associate with the polypeptide structure, which results in an elevation of the commonly measured shrinkage temperature.

Discussion

N. Ramanathan (*Central Leather Research Institute, Madras, India*): If shrinkage is due to uncoiling of the collagen helix, a breakage in the crosslinks holding the three chains together would occur. In this case, it is difficult to see how a recovery of the specimen on cooling in water, such as found in formaldehyde tanned leather, takes place to give us almost the same leather we started with, because if the triple-helix is split up into its constituent chains during recovery, the chains

will have to join up exactly as before. Is it not possible for a disorientation of the collagen helices to take place during shrinkage and a reorientation parallel to the fiber axis during recovery?

L. P. Witnauer: The transition of collagen helices to random coils has been demonstrated to be reversible. The degree of reversibility, of course, would depend upon the experimental conditions employed. Covalent crosslinks would aid in reestablishing the original configuration. Physical examination using optical rotation, dilatometry, x-ray diffraction, and electron microscopy has shown that the rearrangement is intrahelical rather than interhelical.

G. N. Ramachandran (*University of Madras, India*): The problem of shrinkage and the conditions required for it are interesting from the structural point of view. It would appear that the existence of other molecules capable of holding hydrogen bonds in the intermediate state is necessary for the collagen structure to go over from the regular to the folded structure in the shrunken state. This is similar to the α-β transformation of keratin where the extended β-structure is taken up only in the presence of water vapor. It would be interesting to try shrinkage experiments on collagen in nonpolar liquids.

N. Ramanathan: It is implied that only the portion of diluent outside the crystallites is effective in altering the melting temperature at least as far as the thermodynamic relation goes. At the same time, a diluent is effective only when it associates in some way with the helical polypeptide coils of collagen. This means that, in this context, diluents such as ethylene glycol, formamide, and phenol associate with collagen only in the noncrystalline regions and that any effect caused by these on collagen as such would spring from the changes in the noncrystalline regions, the crystallites remaining unaffected. Is this correct?

L. P. Witnauer: A diluent will be effective only if it is mixable with polypeptide chains in the disordered regions. At the melting point, the chemical potentials of the two phases must be equal. If the diluent did not interact with the polypeptide chains in the disordered regions, the melting temperature of the crystalline region would remain invariant with composition as a consequence of the phase rule.

N. Ramanathan: It is not clear how on immersion in water, the vegetable-tanned leather shows a higher shrinkage temperature than that of unmodified hide. Will not the diluent action of vegetable tannin be present after immersion in water also?

L. P. Witnauer: The dilution action of vegetable tannin is present after immersion in water; however, its effect on lowering the shrinkage temperature is greatly overshadowed by the role that it plays in reducing the amount of water that can act as a diluent.

N. Venkateswara Rao (*Loyola College, Madras, India*): In your paper, the shrinkage temperatures are identified with the melting temperatures reported by Flory for the collagen-ethylene glycol mixtures. Flory has shown that when the melted collagen is cooled, there is a partial decrease in volume indicating partial reversion to crystallinity and that the melting temperature is reproducible. May we know whether there was partial restoration of the original length in the shrunken hide after cooling and whether the shrinkage temperature was reproduced?

L. P. Witnauer: There is a partial restoration of the original length in the shrunken hide after cooling. No attempt was made to determine the melt temperature of previously shrunken hide by a shrinkage measurement. Long periods of equilibration at temperatures immediately below the melt temperature would be required in order to obtain the largest and most stable crystallites possible.

G. N. Ramachandran: The fact that the shrinkage (or melting) temperature is not a function of the structure (of the collagen protofibril) alone, but is also a function of the solvent is a fact to which adequate attention has not been paid by the theorists. Thus, as stated by Witnauer, the extrapolated dry shrinkage temperature for mammalian collagen is near 150°, but when heated above this temperature, it does not shrink, but only decomposes. Shrinkage requires the existence of molecules like water to which the hydrogen bonds which stabilize the structure can be transferred. Different molecules have varying capacities for forming such bonds. Dr. Witnauer's data (Figs. 4 and 5) suggest that formamide is more effective than water than phenol, as may be expected from their molecular structure. If suitable salts are added to water, the shrinkage temperature can be lowered to below room temperature, but the nature of the shrunk collagen is identical in all cases and the structure is recovered at the molecular level on removing the agent which produced shrinkage. [Ramachandran, G. N., *Recent Advances in Gelatin and Glue Research*, G. Stainsby, Ed., Pergamon Press, London, 1957, p. 32; Santhanam, M. S., *Proc. Indian Acad. Sci.*, **A49**, 215 (1959).]

The work of Witnauer points to the need for measuring shrinkage temperatures not only in water, but in other reagents in order to understand the mechanism of shrinkage fully.

L. P. Witnauer: There is need for much more work along these lines.

G. N. Ramachandran: Are data on shrinkage temperature available for infinite dilution for the reagents used—that is, when the fiber is immersed in phenol or formamide?

L. P. Witnauer: No shrinkage temperature data were obtained with fibers immersed in phenol or formamide.

B. C. Basu (*Central Leather Research Institute, Madras, India*): Could Dr. Witnauer kindly explain why, at lower moisture contents, vegetable tannin acts as a diluent whereas at higher moisture content it acts as a cross-bonding agent?

In this respect, I like to suggest that it is not the vegetable tannin but the small molecular size polyphenols or phenol-carboxylic acids which are present in vegetable tanned leathers that act as a diluent. At higher moisture content, or with the removal of water solubles, the above mentioned diluents are removed, resulting in the reformation of some original crosslinks of collagen and hence the higher shrinkage temperature value was obtained.

L. P. Witnauer: Vegetable tannin acts as a diluent at all moisture levels. Its major role at the higher moisture levels is to greatly reduce the amount of water that can associate with the polypeptide chains thereby reducing the diluent action of the water. As pointed out, vegetable tannin is a rather complex molecular system; however, under the experimental conditions employed (equilibration of tanned specimens in an atmosphere of water vapor), the water solubles would not have been removed from the specimen.